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PRINCIPAL INVESTIGATOR: Allan D. Hess, Ph.D.

CONTRACTING ORGANIZATION: The Johns Hopkins University
School of Medicine
Baltimore, Maryland 21205-2196

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Allan D. Hess, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Johns Hopkins University School of Medicine
Baltimore, Maryland 21205-2196

E-MAIL:
Adhess@jhmi.edu

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13. ABSTRACT ***(Maximum 200 Words)***

Systemic chemotherapy including high dose chemotherapy with autologous stem cell rescue frequently induces responses in women with metastatic breast cancer. In spite of the substantial response rate, most women will have recurrent disease. Recurrence and progression of breast cancer is thought to be due to chemotherapy resistant tumor cells. The immune system including cytolytic T lymphocytes can effectively target and kill chemotherapy resistant tumor cells. The findings that tumor associate antigens, particularly antigens derived from the Her-2/neu oncogene can be recognized in breast cancer suggests that strategies to enhance immune recognition of these tumor antigens may provide a therapeutic benefit. Recent studies indicate that the N-terminal flanking region of the peptide from the invariant chain termed CLIP has superagonistic properties that can augment the immune response to nominal peptide antigens. The central hypothesis of this proposal is that the N-terminal flanking region of CLIP can augment the immunogenicity of cryptic "self" epitopes from tumor associated antigens such as Her-2/neu. Chimeric constructs of Her-2/neu peptide with the N-terminal flanking region of CLIP will be utilized to determine whether vaccination with this construct can augment the immune response to this antigen. Analysis of the immune response to these peptides will include antibody production, CD4+ and CD8+ dependent cellular immunity (lymphoproliferation, cytokine production) and cytolytic T cell function. Most importantly, studies will be undertaken to determine whether this strategy utilizing the chimeric constructs of the Her-2/neu peptide antigen can induce significant antitumor immunity in vivo. These studies will identify novel strategies to enhance the immunogenicity of tumor peptides particularly related to breast cancer. Importantly, mobilization of an effective immune response to tumor associated antigens will certainly enhance therapeutic strategies in the treatment of breast cancer.

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Introduction:

Breast Cancer is an increasingly common malignancy representing 30 % of all cancers in women. Although conventional systemic chemotherapy or high dose chemotherapy or high dose chemotherapy with autologous stem cell rescue can induce remissions in a significant number of patients, complete curative responses are uncommon and nearly all patients die of progressive disease within 3 years.[1-6] Tumor recurrence and progression are thought to be due to the persistence of clones resistant to chemotherapy. Therefore, strategies that target these chemotherapy resistant tumor cells are required to have a significant impact on the treatment of breast cancer. Recent studies suggest that immunologic approaches may have meaningful clinical impact targeting chemotherapy resistant tumor cells[7]. In this regard, development of strategies to maximize antitumor immunity require augmenting immune mechanisms that specifically recognize tumor associated antigens.[8, 9, 10] Recent studies suggest that the Her-2/neu oncogene can act as a tumor associated antigen in breast cancer.[11, 12] The development of vaccine strategies for breast cancer have focused around the findings that there appears to be active immunity to the Her-2/neu protein.[13, 14] Unfortunately, the immune response is weak and in effective largely due to the fact that Her-2-neu is a "self" protein with central tolerance to the immunodominant epitopes leaving only cryptic epitopes to be functionally recognized.[15] The principal focus of the current research project is to augment the immunogenicity of the cryptic epitopes from Her-2/neu inducing significant antitumor immunity. The central strategy of the proposed work is to strategically modify the cryptic epitope peptides from Her-2/neu with an amino acid sequence from the N-terminal flanking region of the invariant chain peptide termed CLIP. Previous studies have demonstrated that the N-terminal flanking region of CLIP has superagonistic properties interacting with the Vβ segment of the T cell receptor increasing the affinity of the MHC class II-peptide-T cell receptor complex.[16, 17] Increasing the affinity of this complex leads to activation of the T lymphocytes capable of recognizing the specific peptide sequence. Thus, the current research proposal seeks to determine whether the immunogenicity of the Her-2/neu peptides can be augmented by the addition of the N-terminal flanking region of CLIP, to characterize the immune response that is induced by immunization with the Her-2/neu chimeric peptide constructs and to determine whether immunization with these peptides elicits a heightened immune response leading to the induction of protective antitumor immunity.

Body:

Initial studies evaluated whether the -KPVSP(M)- sequence from the N-terminal flanking region of CLIP could augment the immunogenicity of the p1171-1185 peptide (sequence – MTLERPKTLSPGKNGV-) from Her-2/neu in a rat breast cancer model (CRL 1662) that expresses c-neu. Tumor cells were loaded with the parent peptide or the chimeric construct. As a control, the tumor cells were loaded with the N-terminal truncated variant of CLIP. The loaded tumor cells were irradiated (5000 R) and injected subcutaneously at 4 sites (2.5 X 10^5 per site) on the back of the animals. The animals were re-vaccinated 14 days later. Two weeks following the second vaccination, the animals were either evaluated immunologically or challenged with viable tumor cells administered intraperitoneally. Vaccination of animals with (data summarized in appended manuscript) tumor cells loaded with the chimeric peptide elicited a potent cytolytic T cell response capable of killing unmodified tumor cells. In comparison, vaccination of animals with tumor cells loaded with either the parent Her-2/neu or the N-terminal truncated variant of CLIP did not result in the induction of any significant cytolytic T cell activity. The specificity of the cytolytic response (in order to determine whether the chimeric peptide was recognized by the cytolytic T cells) was further explored evaluating the ability of the cells from the animals immunized with the chimeric Her-2/neu peptide to kill peptide loaded tumor cells. Loading the tumor cells with the chimeric peptide or the other MHC class II binding peptides (unmodified parent Her-2/neu peptide, the N-terminal truncated CLIP variant) had little, if any, significant effect on the susceptibility of the target cells to killing mediated by the primed effector cells. The frequency of responding cells (largely CD4+ T cells established by flow cytometric analysis of T cell clones; data not presented) was also assessed in limiting dilution. The frequency of responding T cells in animals

vaccinated with the chimeric construct was significantly increased compared to animals vaccinated with the parent peptide (appended manuscript). The lymphocytes from the chimeric construct primed animals also responded to the parent peptide. In contrast, the response of the lymphocytes from animals vaccinated with the parent peptide was minimal.

Vaccination of the animals with the tumor cells loaded with the chimeric Her-2/neu peptide resulted in the induction of protective antitumor immunity. As shown in Figure 1, animals vaccinated with the chimeric Her-2/neu tumor cell preparation were resistant to live tumor cell challenge. Vaccination with the chimeric peptide resulted in 50% of the animals being resistant to challenge with 3 X 10^6 live tumor cells. Comparatively, animals immunized with the tumor cells loaded with the parent peptide or the N-terminal truncated variant of CLIP all succumbed to tumor growth by day 15. At a lower tumor cell challenge dose (3 X 10^5), 75% of the animals vaccinated with the chimeric Her-2/neu tumor cell preparation survived. There was, at best, only a marginal effect when the animals were immunized with the tumor cells loaded with the parent peptide or the N-terminal truncated variant of CLIP. Nevertheless, these animals succumbed to tumor challenge by day 32.

The efficacy of the chimeric Her-2/neu peptide construct was also evaluated utilizing peptide loaded dendritic cells. These studies, however, would only be effective if an MHC class I peptide could be identified that elicited an MHC class I restricted cytolytic T cell response and was expressed on the tumor cells. Recent studies have identified the binding motif for MHC class I molecules in F344 rats.[18] Based on computer modeling, 6 potential MHC class I binding peptide candidates were identified. One peptide (p554-562) was found to be immunogenic. As demonstrated in Figure 2A, immunization of animals with dendritic cells (5 x 10^4 cells per site, 4 sites) loaded with this MHC class I binding peptide elicited a cytolytic T cell response. Spleen cells from these animals were capable of killing peptide loaded PHA blast cells but demonstrated no specific killing of unloaded PHA blast cells. More importantly, as shown in Figure 2B, spleen cells from the immunized animals were able to kill unmodified tumor cells clearly indicating that this peptide is presented by this tumor cell line. These findings were confirmed in three additional animals immunized with the peptide loaded dendritic cells.

Based on these findings, dendritic cells were loaded with combinations of the MHC class I binding peptide and the parent or the chimeric MHC class II binding peptides. Additionally, the dendritic cells were also loaded with the truncated variant of CLIP containing the N-terminal flanking region. Animals were vaccinated with the peptide loaded dendritic cells (5 X 10^4) cells/site at 4 sites X 2; 14 days apart). Subsequent to the last immunization (14 days) the animals were evaluated for cytolytic T cell function and for the induction of protective antitumor immunity. As shown in Figure 3, a potent cytolytic T cell response could only be demonstrated in animals immunized with the dendritic cells loaded with the MHC class I Her-2/neu peptide and the chimeric Her-2/neu construct. These cytolytic T cells belonged to the CD 8+ T cell subset as confirmed in depletion experiments (Percent killing at a 30:1 effector to target ratio: Mean +/- S.E.M., n=3, Control, 52.3 +/- 4.9, CD 4 depleted, 48.3 +/- 3.7, CD 8 depleted, 2.6 +/- 2.8). Weak or modest cytolytic T cell responses could be demonstrated for the animals vaccinated with the dendritic cells loaded with the MHC class I Her-2/neu peptide plus the parent MHC class II Her/2/neu peptide or with dendritic cells loaded with only the MHC class I peptide from Her-2/neu. In accord with these results are the findings that the animals vaccinated with the peptide from Her-2/neu plus the chimeric construct presented on dendritic cells were resistant to live tumor challenge (Figure 4). Comparatively, all other groups vaccinated with dendritic cells variably loaded with the different peptide combinations succumbed to tumor challenge.

Studies were also undertaken to evaluate whether protective antitumor immunity required both CD4+ and CD8+ T cells. Spleen cells from the vaccinated animals and resistant to tumor challenge were harvested and fractionated into the CD4+ and CD8+ T cell subsets prior to adoptive transfer into naive F344 rats. The rats were challenged with 3 X 10^5 live tumor cells. As shown in Figure 5, animals receiving unfractionated spleen cells or the combination of CD4+ plus CD8+ T lymphocyte subsets were

resistant to tumor challenge. Animals receiving just the isolated CD4+ or CD8+ T cell subsets succumbed to tumor challenge.

Taken together, studies conducted during the current year of funding demonstrate that the immunogenicity of a cryptic peptide from the oncogene Her-2/neu could be augmented by the addition of the N-terminal flanking region of CLIP. Immunizing animals with the chimeric peptide either presented on tumor cells or on dendritic cells loaded with an immunogenic MHC class I binding peptide from Her-2/neu elicited protective antitumor immunity in a rat model of breast cancer. These data are in accord with the initial hypothesis of this proposal. Subsequent studies plan to further elucidate the cellular immunological basis for the protective antitumor immune response specifically evaluating the V region repertoire, cytokine profile and specificity of the effector T cells as outlined in the original proposal. Additionally, we plan to utilize these strategies to determine whether significant antitumor immunity can be induced in animals with actively growing tumor.

Key Research Accomplishments:

- demonstrated that the immunogenicity of a cryptic Her-2/neu peptide can be augmented by the addition of the N-terminal flanking region of CLIP
- demonstratred that immunization of animals with the chimeric Her-2/neu peptides can elicit a potent cytolytic T cell response
- demonstrated that the chimeric Her-2/neu peptide is effective when presented on tumor cells or in the context of dendritic cells along with an MHC class I immunogenic peptide from Her-2/neu
- demonstrated that effective antitumor immunity in this setting requires both CD4+ and CD8+ T lymphocytes
- demonstrated that vaccination with the chimeric Her-2/neu peptide elicits protective antitumor immunity

Reportable Outcomes:

Studies conducted during the first year of funding have been submitted for publication to Cancer Research and the manuscript entitled "The N-Terminal Flanking Region of the Invariant Chain Peptide Augments the Immunogenicity of a Cryptic Epitope from the Her-2/neu Tumor Associated Antigen" is appended.

Conclusions:

The present project is based on the hypothesis that the N-terminal flanking region of the invariant chain peptide termed CLIP has superagonistic properties interacting the T cell receptor and the MHC class II molecule at the binding site for the bacterial superantigen, staphylococcal enterotoxin B. The current studies tested the hypothesis that this N-terminal segment of CLIP can augment the immunogenicity of cryptic "self" tumor associated antigens from Her-2/neu. The results indicate that the immunogenicity of a cryptic peptide from Her-2/neu can be augmented by adding the N-terminal flanking region of CLIP. The chimeric peptides elicit potent antitumor cytolytic T cell activity against a Her-2/neu+ tumor rat model leading to the induction of systemic protected antitumor immunity. The chimeric peptide was effective at inducing an antitumor immune response when presented either on tumor cells or on dendritic cells in concert with an immunogenic MHC class I peptide from Her-2/neu. These results clearly suggest that modification of peptides from tumor associated antigens by creating chimeric constructs containing N-terminal flanking region of CLIP is an effective strategy to augment the immunogenicity of these peptide antigens. Most importantly, however, will be the studies conducted in

the next year of funding to determine whether these strategies can effectively augment antitumor immunity in host with actively growing tumors.

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Appendices

A. Figures 1-5

B. Manuscript submitted for publication

Figure 1 Vaccination with chimeric Her-2/neu peptide loaded tumor cells induced protective antitumor immunity

F344 rats were vaccinated (2X, 14 days apart) with peptide loaded, irradiated (5000R) CRL 1666 tumor cells. Included in the panel of peptides were the parent p1171-1185 peptide, the chimeric construct and the truncated variant of CLIP containing the N-terminal flanking region. Fourteen days following the second vaccination, the animals were challenged with live tumor cells administered intraperitoneally.

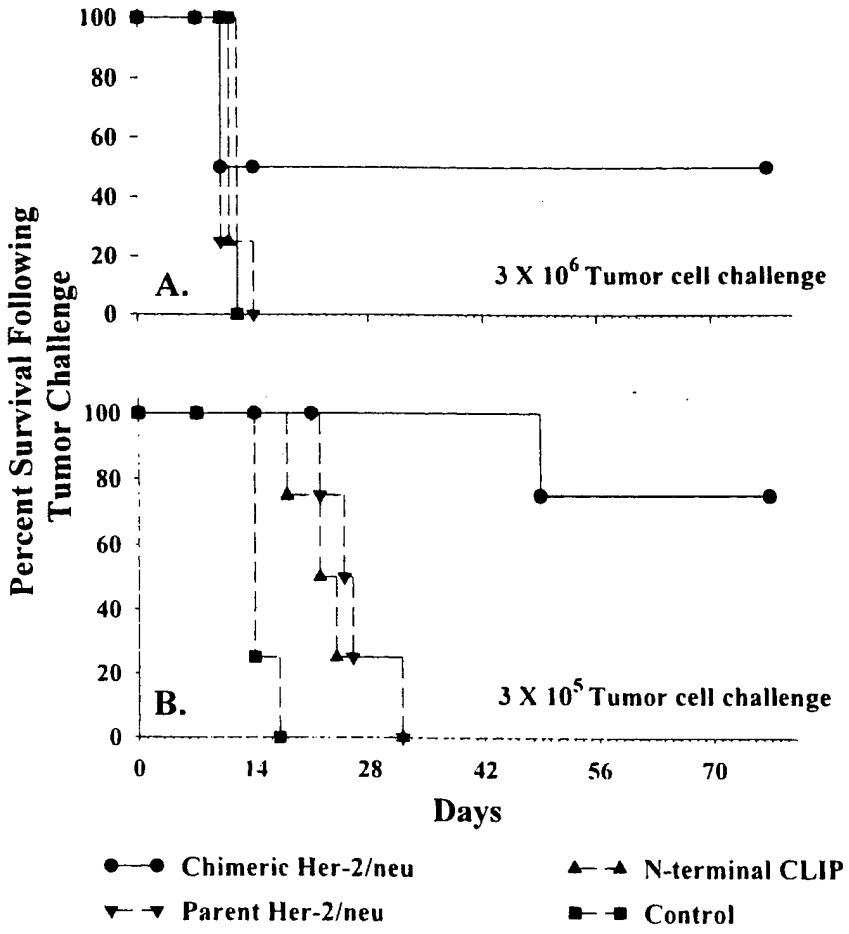


Figure 2A, B Induction of a cytolytic T cell response to a Her-2/neu MHC class I restricted peptide

F344 rats were immunized intradermally (2X, 14 days apart) with dendritic cells (5 x 10^4 cells/site, 4 sites) loaded with the MHC class I binding peptide (p544-562) from Her-2/neu. Splenic T lymphocytes were harvested 14 days later and evaluated for their ability to kill peptide loaded PHA blast cells (A) or unmodified CRL 1666 tumor cells (B).

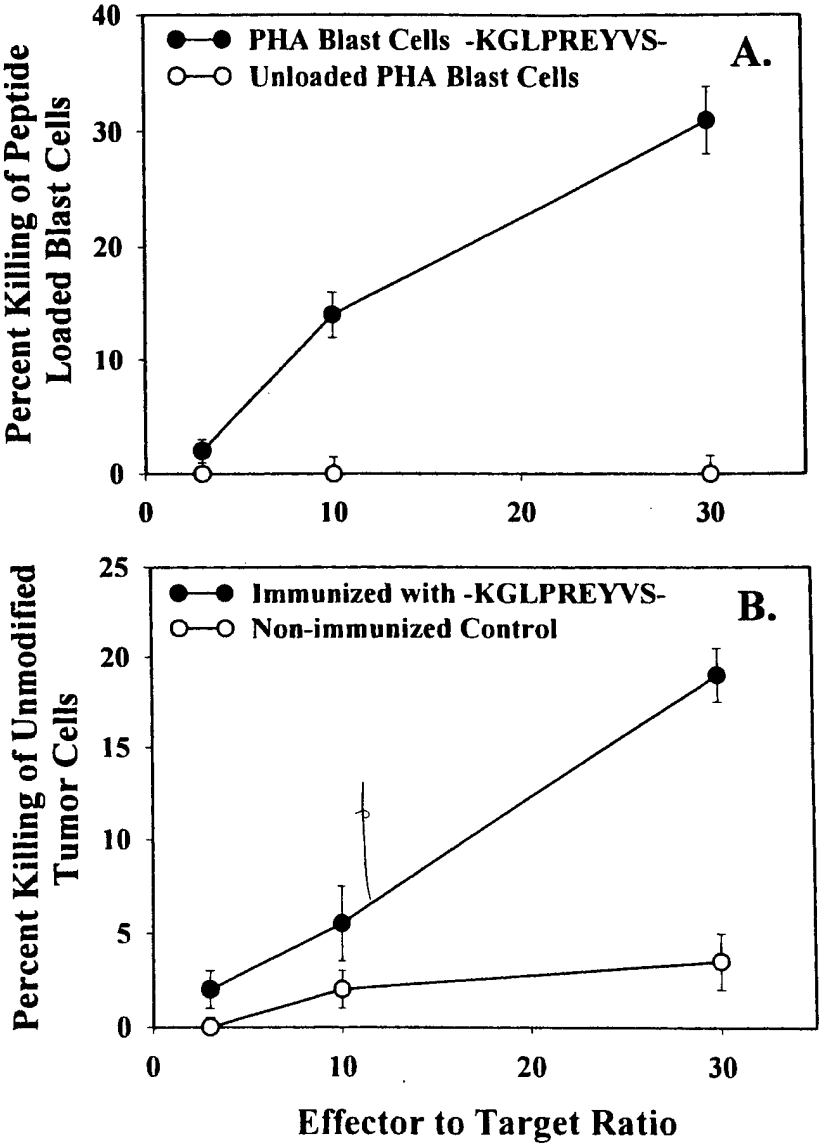


Figure 3 In Vitro targeting of unmodified tumor cells after vaccination with Her-2/neu peptide loaded dendritic cells

F344 rats were vaccinated intradermally twice, 14 days apart with peptide loaded dendritic cells (5×10^4 cells/site, 4 sites). Peptides included the p 1171-1185 parent, the chimeric construct, the N-terminal CLIP variant and the MHC class I binding peptide (p544-562) from Her-2/neu. Peptides were loaded singly or in various combinations. Subsequent (14 days) to the last vaccination, splenic T lymphocytes were harvested and evaluated for their ability to kill unmodified CRL 1666 tumor cells.

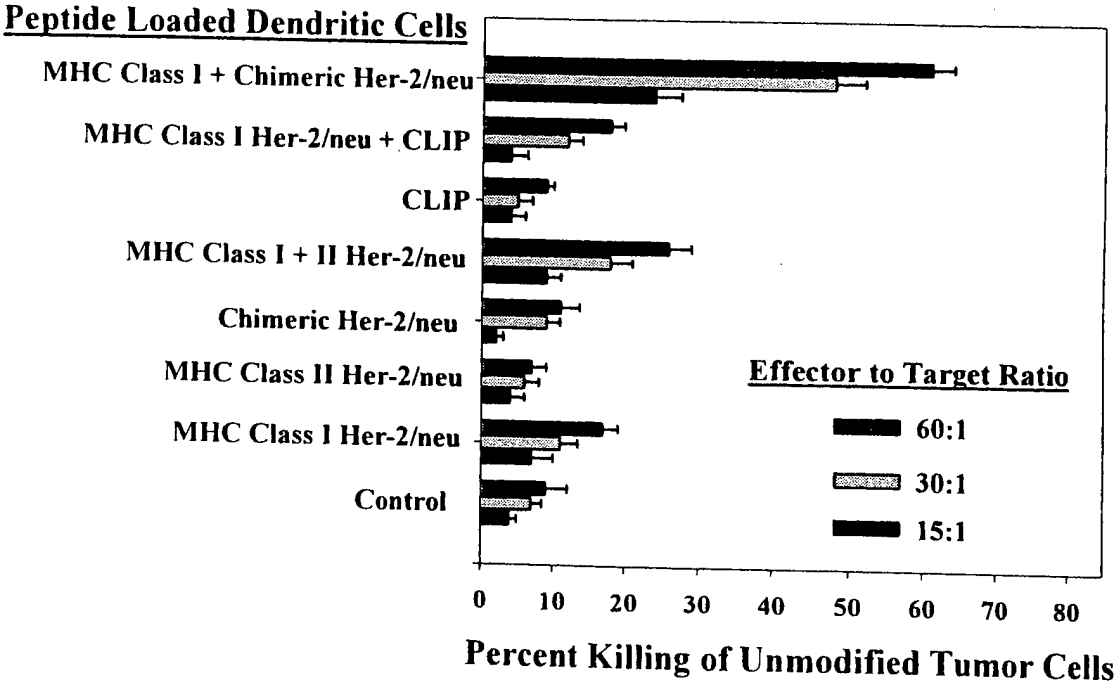


Figure 4 Vaccination with Her-2/neu peptide loaded dentritic cells induces protective antitumor immunity

F344 rats were vaccination intradermally (2X, 14 days apart) with dendritic cells (5 x 10^4 cells/site, 4 sites) that were loaded singly or in various combinations with the MHC class I (p554-562) and class II (p 1175-1185) Her-2/neu peptides, the chimeric construct or the N-terminal CLIP variant. Fourteen days later, the animals were challenged with live tumor (3 x 10^5 CRL 1666 cells) administered intraperitoneally.

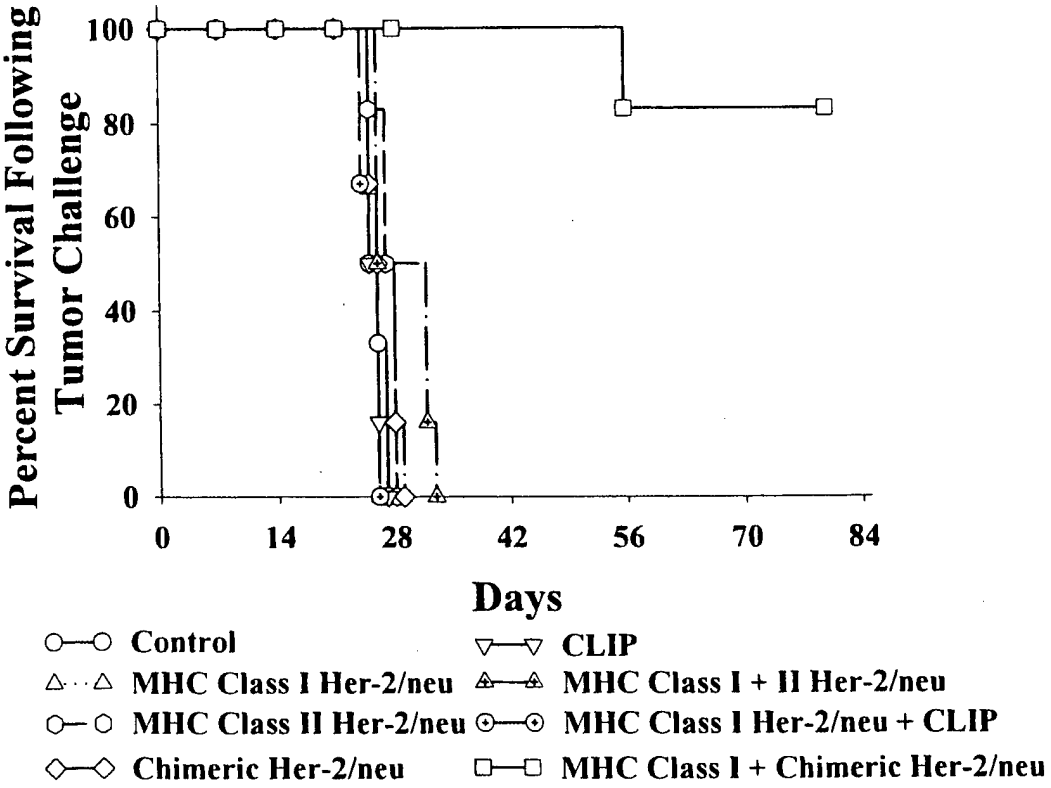
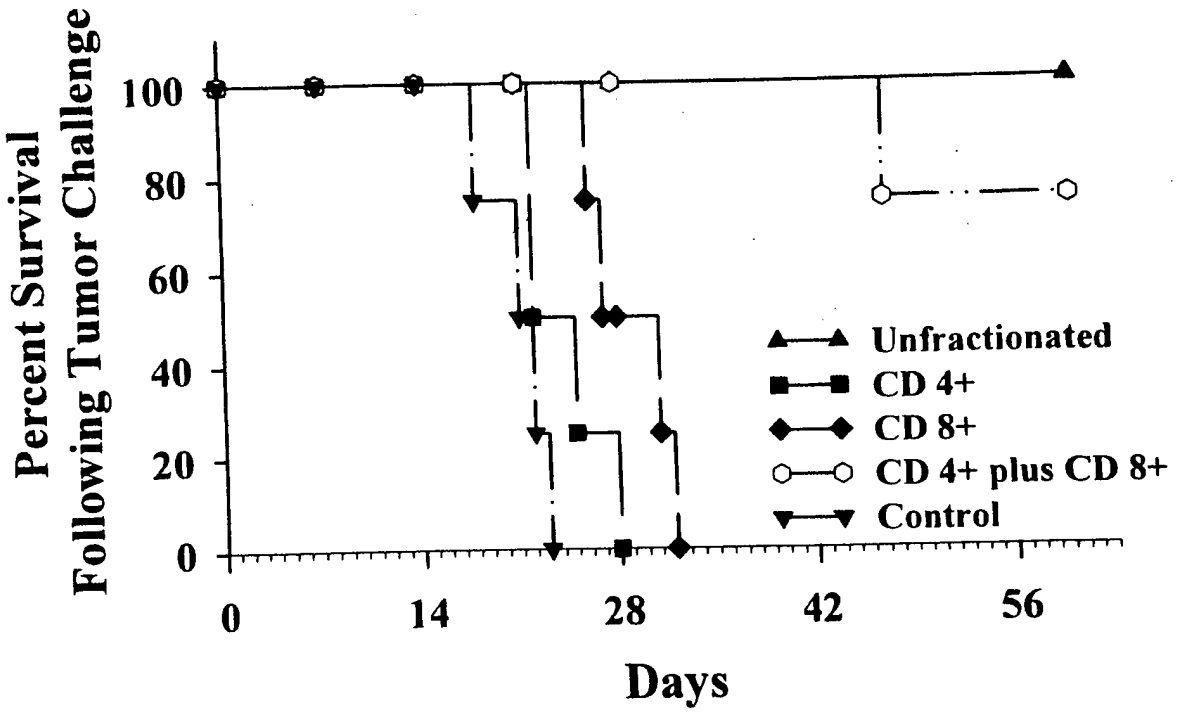


Figure 5 Effective antitumor immunity requires both CD4+ and CD8+ T cells

Animals were vaccinated (2X) intradermally with dendritic cells (5 x 10 4 cells/site, 4 sites) loaded with the MHC class I (p554-562) and the chimeric (p1171-1185) construct. Fourteen days after the last vaccination, the splenic T lymphocytes were harvested and the CD8+ and CD4+ subsets isolated by immunomagnetic bead separation. The cells were adoptively transferred into secondary F344 recipients (unfractionated, isolated CD4+ and CD8+ subsets: 30 x 10^6 cells per recipient: recombined subsets, 15 x 10^6 of each per recipient). Following (1 day) the adoptive transfer, the animals were challenged with 3 x 10^5 viable CRL 1666 tumor cells.



The N-terminal Flanking Region of the Invariant Chain Peptide Augments the Immunogenicity of a Cryptic Epitope from the Her-2/neu Tumor Associated Antigen[1]

Allan D. Hess, Christopher Thoburn,

Louis Horwitz, Weiran Chen and Elsken Van der Wall[2]

Division of Immunology and Hematopoiesis
Department of Oncology, The Johns Hopkins University
Bunting and Blaustein Cancer Research Building
1650 Orleans Street, Baltimore, MD 212131

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Correspondence should be addressed to:

Allan D. Hess, Ph.D.
Bunting-Blaustein Cancer Research Building,
1650 Orleans Street, Room 489
Baltimore, Maryland 21231
Telephone (410)955-8784
Facsimile (410) 502-7163
e-mail: adhess@jhmi.edu

[1]This work was supported by grants AI 24319, CA 82853 and CA 15396 from the National Institute of Health and Grant USAMRMC DAMD17-99-1-9238 from the Department of Defense

[2]Present address
Department of Medical Oncology
Free University Hospital, De Boelelaan 1117
1081 HV Amsterdam
The Netherlands

Abstract

The N-terminal flanking region of the invariant chain peptide termed CLIP, has superagonistic properties interacting with the T cell receptor and the MHC class II molecule at the binding site for the bacterial superantigen, Staphylococcal enterotoxin B (SEB). The present studies explored the hypothesis that the N-terminal segment of CLIP can augment the immunogenicity of cryptic "self" tumor-associated antigens. A chimeric construct of a peptide from the oncogene, Her-2/neu containing the N-terminal flanking region of CLIP elicited potent antitumor activity against a Her-2/neu positive tumor in a rat model system. Comparatively, the unmodified parent peptide was ineffective. The induction of effective antitumor immunity, however, required presentation of the chimeric peptide construct on irradiated tumor cells or the peptide construct in concert with an MHC class I peptide from Her-2/neu.

Introduction

During the past several years, evidence has accumulated indicating that tumor cells express antigens that can be recognized by the immune system.[1-3] These tumor-associated (TA) antigens include normal "self" proteins that are over expressed due to gene amplification,[3,4] however, the immune response to these antigens is weak and ineffective.[5,6] The host is tolerant to the immunodominant epitopes of these antigens leaving only cryptic epitopes to be functionally recognized.[7] The weak immune responses to the cryptic epitopes of tumor antigens appears to be related to the low affinity of the peptide antigens for their presenting MHC molecule resulting in poor presentation of the MHC-peptide ligands to T cells.[8] Augmenting the immunogenicity of these TA antigens is a critical step in enhancing vaccine strategies designed to elicit the induction of antitumor immunity.

Characterization of the effector T cells in the experimentally induced autoaggression syndrome termed autologous/syngeneic graft-vs-host disease (GVHD), reveals a unique mode of antigen recognition that augments recognition of nominal antigenic peptides.[9] This autoaggression syndrome can be induced in man and in rodents by administering Cyclosporine after autologous or syngeneic bone marrow transplantation and is associated with the development of a highly restricted repertoire of autoreactive T cells that promiscuously recognize MHC class II determinants.[10-12] MHC class II recognition occurs even in the absence of the classical CD 4 cell surface restriction element. Recent studies reveal that pathogenic syngeneic GVHD effector T cells recognize a peptide from the MHC class II invariant chain, termed CLIP, presented in the context of MHC class II antigens.[10-14] Most importantly, there appears to be a

functional interaction between the Vβ component of the T cell receptor (TcR) and the N-terminal flanking region of CLIP that extends beyond the peptide binding domain of MHC class II. This superagonistic interaction which occurs at or near the binding site for the staphylococcal enterotoxin B (SEB) superantigen, appears to increase the affinity of the TcR: MHC class II: peptide complex.[10-14]

The present studies explore the hypothesis that the N-terminal flanking region of CLIP can augment the immunogenicity of cryptic "self" TA antigens. The results reveal that vaccination of animals with a chimeric construct of a cryptic epitope from the Her-2/neu oncogene and the N-terminal flanking region of CLIP elicited a potent cytolytic T cell response and the induction of protective antitumor immunity. The induction of effective antitumor immunity, however, requires either presentation of the chimeric peptide construct on irradiated tumor cells or the peptide construct in concert with an MHC class I binding peptide from Her-2/neu presented on dendritic cells.

Materials and Methods

Animals

Fischer (F344) strain rats, 4-6 weeks of age were purchased from Charles River, Inc. (Wilmington, MA). The animals were kept in sterile microisolator cages and fed food and water *ad libitum*. The animals were challenged with tumor intraperitoneally. For the adoptive transfer studies, the rats were pretreated with cyclophosphamide (100 mg/kg) 1 day prior to receiving immune spleen cells and tumor challenge. Four to six animals were used for each experimental group.

Tumor Cells

The breast cancer cell line, CRL 1666, derived from F344 strain rats was purchased from the American Type Culture Collection (ATCC). The tumor cells express MHC class I and II antigens and express c-neu as detected by mouse anti-rat c-neu monoclonal antibody (Ab-4; Oncogene Research Products, Calbiochem, Cambridge, MA). The cell line was maintained *in vitro* in McCoy's 5A tissue culture medium (Grand Island biological Co. Gibco, Grand Island, NY) supplemented with 10% fetal calf serum. The cells were washed three times in tissue culture prior to use in *in vitro* assays or *in vivo* intraperitoneal challenge.

Effector Cell Isolation

Spleens from control and experimental animals were harvested and passed through a wire mesh screen to obtain a single cell suspension. The mononuclear cell fraction was isolated by Ficoll-Hypaque density centrifugation and further fractionated by nylon wool columns to enrich for T lymphocytes as previously described.[12-15] The CD8+ and CD4+ T lymphocyte subsets were isolated by immunomagnetic bead separation using the anti-rat CD4 and CD8 murine monoclonal antibodies (Serotec, Bioproducts for Science, Indianapolis, IN) as described previously.[15] The purity of the population was confirmed flow cytometrically by staining the cells with monoclonal antibodies to rat CD4 and CD8 cell surface determinants and counter staining with rat adsorbed, fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse IgG (Sigma Chemical Co., St. Louis, Mo.). Cells stained with normal mouse serum and

counterstained with the FITC anti-mouse IgG served as the control.

Dendritic cells were isolated from spleen cells based on differential plastic adherence as previously described.[16] Briefly rat spleen cells were incubated for 2 hours in tissue culture flasks. The flasks were rinsed thoroughly with tissue culture medium. After 18 hours of incubation, the dendritic cells that detached from the plastic tissue culture flasks were harvested and washed in tissue culture medium. The cells were confirmed to be dendritic cells by their potent stimulatory activity of allogeneic lymphocytes in mixed lymphocyte reactions and by expression of OX62 (Pharmigen, San Diego, CA) the rat dendritic cell marker, assessed flow cytometrically.

Immunological Assessment

Killing was assessed using a ^{3}H-thymidine-based assay (JAM) as described by Matzinger, that measures DNA fragmentation and cell death.[17] The target cells (PHA blast cells, tumor cells; 5-10 x 10^{6}) were pulsed with 2.5 μ Ci/ml of ^{3}H-thymidine for 18 hours and washed three times before assay. Graded numbers of the effector T cells and the target cells (5 x 10^{3}) were coincubated for 4 hours before harvest.

The frequency of the responding T cells after vaccination was assessed utilizing a limiting dilution technique as previously described.[12-15] Briefly, splenic lymphocytes were cultured at limiting dilution utilizing irradiated syngeneic spleen cells loaded with parent or chimeric Her-2/neu peptides (MHC class II binding) as APC's in complete tissue culture medium containing IL-2 (10U/ml). Positive wells were visually scored

after 14 days of culture and the clones were expanded by restimulation (every 7 to 10 days) with irradiated peptide pulsed syngeneic spleen cells (2×10^4 cells/macrotiter well) for Vβ TcR gene usage as previously described.[12, 15]

Peptides

The sequences of the peptides principally utilized in the present studies are given in Table 1 and include the truncated variant of CLIP containing the N-terminal flanking region (p 86-100), the fluoresceinated derivative of p86-100, (for binding studies), the parent MHC class II binding Her-2/neu peptide (p1171-1185) described by Dsis et al[18,19] and the chimeric derivative containing the N-terminal flanking region of CLIP.

Recent studies have elucidated the binding motif for Lewis/F344 strain rats.[20] The peptide ligands are nonamers that contain a hydrophobic leucine anchor residue at position 3 and a carboxyl terminal serine anchor residue. Computer modeling of the rat Her-2/neu amino acid sequence revealed 5 potential candidates that could bind to Lewis/F344 MHC class I molecules. Initial studies revealed one sequence (p554-562) that elicited a cytolytic T cell response (described in the results) and is listed in Table 1. The other peptides (p377-385, p403-411, p439-447, p790-798 and p1105-1113) were either weakly or non-immunogenic. The peptides, chemically synthesized and purified by high pressure liquid chromatography, were obtained from Quality Controlled Biochemicals (Hopkinton, MA). The peptides (>92% purity) were diluted to 10 μM in RPMI 1640 prior to loading as previously described.[10,11,13] Previous dose-response

studies revealed that maximal saturation was achieved by pretreating the target cells with 1 μM peptide.

Results

Initial studies evaluated whether the -KPVSP(M)- sequence from the N-terminal flanking region of CLIP could augment the immunogenicity of the p1171-1185 peptide from Her-2/neu. Tumor cells were loaded with the parent peptide or the chimeric construct. As a control, the tumor cells were loaded with the N-terminal truncated variant of CLIP. The loaded tumor cells were irradiated (5000 R) and injected subcutaneously at 4 sites (2.5×10^5 per site) on the back of the animals. The animals were re-vaccinated 14 days later. Two weeks following the second vaccination, the animals were either evaluated immunologically or challenged with viable tumor cells administered intraperitoneally. A representative (1/4) experiment is presented in Figure 1. Vaccination of animals with tumor cells loaded with the chimeric peptide elicited a potent cytolytic T cell response capable of killing unmodified tumor cells. In comparison, vaccination of animals with tumor cells loaded with either the parent Her-2/neu or the N-terminal truncated variant of CLIP did not result in the induction of any significant cytolytic T cell activity. The specificity of the cytolytic response (in order to determine whether the chimeric peptide was recognized by the cytolytic T cells) was further explored evaluating the ability of the cells from the animals immunized with the chimeric Her-2/neu peptide to kill peptide loaded tumor cells. As shown in Figure 2, loading the tumor cells with the chimeric peptide or the other MHC class II binding peptides (unmodified parent Her-2/neu peptide, the N-terminal truncated CLIP variant) had little, if any, significant effect on the susceptibility of the target cells to killing mediated by the primed effector cells.

The frequency of responding cells (largely CD4+ T cells established by flow cytometric analysis of T cell clones; data not presented) was also assessed in limiting dilution. As shown in Figure 3A, B, the frequency of responding T cells in animals vaccinated with the chimeric construct was significantly increased compared to animals vaccinated with the parent peptide. The lymphocytes from the chimeric construct primed animals also responded to the parent peptide. In contrast, the response of the lymphocytes from animals vaccinated with the parent peptide was minimal.

Vaccination of the animals with the tumor cells loaded with the chimeric Her-2/neu peptide resulted in the induction of protective antitumor immunity. As shown in Figure 4, animals vaccinated with the chimeric Her-2/neu tumor cell preparation were resistant to live tumor cell challenge. Vaccination with the chimeric peptide resulted in 50% of the animals being resistant to challenge with 3×10^6 live tumor cells. Comparatively, animals immunized with the tumor cells loaded with the parent peptide or the N-terminal truncated variant of CLIP all succumbed to tumor growth by day 15. At a lower tumor cell challenge dose (3×10^5), 75% of the animals vaccinated with the chimeric Her-2/neu tumor cell preparation survived. There was, at best, only a marginal effect when the animals were immunized with the tumor cells loaded with the parent peptide or the N-terminal truncated variant of CLIP. Nevertheless, these animals succumbed to tumor challenge by day 32.

The efficacy of the chimeric Her-2/neu peptide construct was also evaluated utilizing peptide loaded dendritic cells. These studies, however, would only be effective if an MHC class I peptide could be identified that elicited an MHC class I restricted cytolytic T cell response and was expressed on the tumor

cells. Recent studies have identified the binding motif for MHC class I molecules in F344 rats.[20] Based on computer modeling, 6 potential MHC class I binding peptide candidates were identified. One peptide (p554-562) was found to be immunogenic. As demonstrated in Figure 5A, immunization of animals with dendritic cells (5 x 10^4 cells per site, 4 sites) loaded with this MHC class I binding peptide elicited a cytolytic T cell response. Spleen cells from these animals were capable of killing peptide loaded PHA blast cells but demonstrated no specific killing of unloaded PHA blast cells. More importantly, as shown in Figure 5B, spleen cells from the immunized animals were able to kill unmodified tumor cells clearly indicating that this peptide is presented by this tumor cell line. These findings were confirmed in three additional animals immunized with the peptide loaded dendritic cells.

Based on these findings, dendritic cells were loaded with combinations of the MHC class I binding peptide and the parent or the chimeric MHC class II binding peptides. Additionally, the dendritic cells were also loaded with the truncated variant of CLIP containing the N-terminal flanking region. Animals were vaccinated with the peptide loaded dendritic cells (5 X 10^4) cells/site at 4 sites X 2; 14 days apart). Subsequent to the last immunization (14 days) the animals were evaluated for cytolytic T cell function and for the induction of protective antitumor immunity. As shown in Figure 6, a potent cytolytic T cell response could only be demonstrated in animals immunized with the dendritic cells loaded with the MHC class I Her-2/neu peptide and the chimeric Her-2/neu construct. These cytolytic T cells belonged to the CD 8+ T cell subset as confirmed in depletion experiments (Percent killing at a 30:1 effector to target ratio: Mean +/- S.E.M., n=3, Control, 52.3 +/- 4.9, CD 4 depleted, 48.3 +/- 3.7, CD 8 depleted, 2.6 +/-2.8). Weak or modest cytolytic T cell responses could be demonstrated for the animals vaccinated with the dendritic cells

loaded with the MHC class I Her-2/neu peptide plus the parent MHC class II Her/2/neu peptide or with dendritic cells loaded with only the MHC class I peptide from Her-2/neu. In accord with these results are the findings that the animals vaccinated with the peptide from Her-2/neu plus the chimeric construct presented on dendritic cells were resistant to live tumor challenge (Figure 7). Comparatively, all other groups vaccinated with dendritic cells variably loaded with the different peptide combinations succumbed to tumor challenge.

Studies were undertaken to evaluate whether protective antitumor immunity required both CD4+ and CD8+ T cells. Spleen cells from the vaccinated animals and resistant to tumor challenge were harvested and fractionated into the CD4+ and CD8+ T cell subsets prior to adoptive transfer into naive F344 rats. The rats were challenged with 3×10^5 live tumor cells. As shown in Figure 8, animals receiving unfractionated spleen cells or the combination of CD4+ plus CD8+ T lymphocyte subsets were resistant to tumor challenge. Animals receiving just the isolated CD4+ or CD8+ T cell subsets succumbed to tumor challenge.

One potential mechanism to account for the heightened immunogenicity of the chimeric peptide construct is that it might have an increased affinity for MHC class II molecules compared to the parent, unmodified peptide. Studies were undertaken to evaluate whether the chimeric peptide construct had a greater affinity than the parent molecule. In order to approach this question, a flow cytometric assay was developed in which PHA blast cells were stained with fluoresceinated CLIP. As shown in Figure 9, both the parent and the chimeric Her-2/neu construct equally inhibited the binding of fluorescent CLIP. This

ability to inhibit fluorescent CLIP binding was virtually identical to the ability of native CLIP to inhibit the binding of the fluoresceinated derivative. Previous studies confirmed binding of the fluorsceinated CLIP to MHC class II since pretreatment of the PHA blast cells with monoclonal antibody to MHC class II determinants inhibited staining whereas anti-MHC class I antibody pretreatment was ineffective.[10, 12]

Discussion

Previous studies in our laboratory revealed that the autoaggression syndrome induced by administering Cyclosporine after syngeneic or autologous bone marrow transplantation is mediated by a highly conserved repertoire of Vβ 8.5+ CD 8+ autoreactive T cells that promiscuously recognize MHC class II determinants.[9,15] Recognition of MHC class II molecules by the autoreactive T cells is dependent on the presentation and recognition of CLIP.[10, 12] This peptide derived from the invariant chain that shepherds the biosynthesis of MHC class II is thought to stabilize MHC class II molecules in the absence of nominal peptides.[21, 22] Essential for the promiscuous recognition of MHC class II, however, is the interaction between the N-terminal flanking region of CLIP that extends beyond the MHC peptide binding groove and the Vβ segment of the TcR at or near the binding site for the superantigen SEB.[12,13,23,24] This interaction could overcome the requisite specificity of the TcR CDR3 domain for the peptide sequence within the peptide binding groove of MHC class II. Presentation of chimeric constructs of irrelevant peptides with the N-terminal flanking region allowed for effective targeting by CLIP reactive T cell clones.[13] Of additional importance in this regard are the findings that the N-terminal fragment of CLIP can promote promiscuous binding of peptides to MHC class II when presented as chimeric constructs.[24] The affinity

of the TcR:peptide:MHC Class II complex appears to be increased by this interaction thus not only potentially explaining the promiscuous specificity of the autoreactive T cells but also the restriction of the repertoire to an SEB responsive subset. Moreover, this interaction appears to override the requirement for the classical cell surface accessory molecule (CD4).[11, 12]

The superagonistic properties of the N-terminal flanking region of CLIP as defined in the autologous/syngeneic GVHD model and its ability to promote promiscuous binding of peptides to MHC class II suggest that this peptide fragment may be able to augment the immunogenicity of nominal peptides including cryptic epitopes from TA antigens. The results from the present studies clearly support this hypothesis. The immunogenicity of an MHC class II binding peptide (p1171-1185) from the rat Her-2/neu oncogene was augmented by the addition of the N-terminal flanking region sequence of CLIP. This Her-2/neu peptide is weakly immunogenic eliciting both antibody and CD 4 T helper responses but required repeated immunizations in adjuvant to evoke this response.[18, 19] Immunization with this parent peptide also failed to induce significant protective antitumor immunity. In the present studies, vaccination with a chimeric construct of this peptide that contained the N-terminal flanking region of CLIP elicited a potent cytolytic T cell response and the induction of protective antitumor immunity. Successful vaccination required presentation of both the N-terminal flanking region and the Her-2/neu peptide. Interestingly, vaccination with the chimeric construct increased the frequency of cells responding to the parent peptide. In accord with our results are recent findings demonstrating that the potency of MHC class II-presented epitopes is increased by linking the peptide to the p77-92 peptide of the invariant chain.[25] Moreover, studies by Naujokas, et al. Suggest that there is an interactive T cell epitope on a flanking region of CLIP that lies

outside of the MHC class II peptide binding domain.[26]

It is important to note, however, that the induction of a successful antitumor response *in vitro* and *in* vivo required either presentation of the chimeric peptide on irradiated tumor cells or the peptide construct in combination with an MHC class I binding peptide from Her-2/neu presented on dendritic cells whereas other combinations or the use of single peptides were ineffective. These data suggest that although the immunogenicity of the p1171-1185 chimeric construct was enhanced (as also evidenced by the results from the limiting dilution studies), there was a requirement for direct recognition of the tumor cell (and presentation of other MHC class I restricted antigens?) or presentation of a Her-2/neu antigen restricted by MHC class I and expressed on the tumor cell in combination with the chimeric construct. The chimeric construct did not appear to be recognized by the cytolytic T cells. These results were initially surprising considering the findings in the autologous/syngeneic GVHD model where the N-terminal flanking region of CLIP allowed for CD 8+ cytolytic T cell targeting of MHC class II.[9-14] Perhaps this unique mode of MHC class II antigen recognition is dependent on the administration of Cyclosporine and its affect on T cell differentiation in the thymus. A number of studies clearly indicate that this drug remarkably alters thymic differentiation and restriction.[27-29] It will be of interest to evaluate the chimeric vaccine strategy after a course of Cyclosporine treatment.

For peptide vaccine strategies to be successful, the tumor cells must express the antigen at the cell surface. In this regard, recent studies by Zaks and Rosenberg demonstrated that immunization with a peptide epitope from Her-2/neu elicited peptide-specific cytolytic T lymphocytes but failed to recognize

Her-2/neu positive tumors.[30] In the present studies, there was recognition and killing of the unmodified tumor cells after immunization with the MHC class I peptide. A potent cytolytic T cell response and the induction of protective antitumor immunity, however, required immunization in conjunction with the chimeric construct. It is important to note that effective immunization with the MHC class I peptide and the chimeric construct presented on dendritic cells elicited a CD 8+ cytolytic T cell response but this response by itself was insufficient for protective antitumor immunity. The adoptive transfer studies clearly show that protective antitumor immunity required priming of the CD 4+ T cell subset, findings that are in accord with several other studies.[31-35] It seems likely that immunization with the chimeric construct primed the CD 4+ T cell subset.

The underlying mechanisms accounting for the heightened immunogenicity of the chimeric construct remains unclear. Competitive inhibition studies did not reveal any significant differences in affinity between the parent peptide or the chimeric construct that might account for the potentiation of immunogenicity.[24, 25] The potential interaction between the N-terminal flanking region and the Vβ segment of the T cell receptor may account for the potentiation of nominal peptide immunogenicity.[12,13,25] Our previous studies suggest that the interaction between the N-terminal flanking region of CLIP and the Vβ segment of the TcR as defined for the autologous/syngeneic GVHD effector T cells occurs at or near the SEB binding site. Such an interaction might skew the repertoire. Preliminary analysis of several clones (17) reveal a skewing of the repertoire to cells expressing Vβ8.5, 11, and 17 elements that confer responsiveness to SEB.[36,37] An extensive analysis, however, is required to show definitive skewing of the repertoire. Additional studies evaluating peptide-TcR-MHC class II interaction at the molecular level also must be undertaken to further

delineate the underlying mechanisms. Nevertheless, the results from the present studies indicate that the N-terminal flanking region of CLIP can augment the immunogenicity of a cryptic epitope from a "self" antigen. Studies are underway evaluation other peptides constructs from Her-2/neu and to determine whether this approach can be effective in animals with actively growing tumors.

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Figure 1 Vaccination with chimeric Her-2/neu peptide loaded tumor cells induces a cytolytic T cell response

F344 rats were vaccinated intradermally (4 sites, twice 14 days apart) with irradiated (5000R) CRL 1666 tumor cells (2.5×10^5) loaded with the p1171-1185 Her-2/neu peptide, the p1171-1185 chimeric construct, the truncated variant of CLIP containing the N-terminal flanking region or the control diluent. Fourteen days following the last vaccination, splenic T cells were harvested and assessed for their ability to kill unmodified CRL 1666 tumor cells.

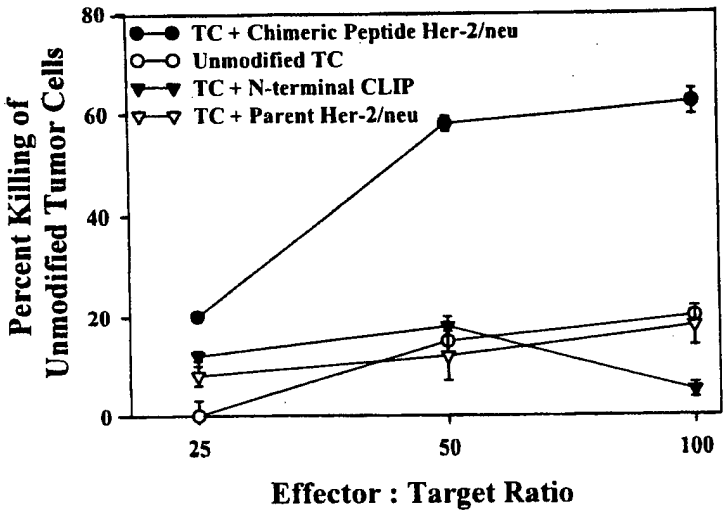

Figure 2 Effect of peptide loading on tumor cell killing

Spleens from animals vaccinated twice with the p1171-1185 chimeric peptide loaded tumor cells were harvested 14 days after the second vaccination. The splenic lymphocytes were isolated by Ficoll-Hypaque density centrifugation and enriched for T cells by nylon wool fractionation. The effector T cells were assessed for their ability to kill tumor cells loaded with the p1171-1185 peptide from Her-2/neu, the p1171-1185 chimeric construct, the N-terminal truncated variant of CLIP or the control diluent.

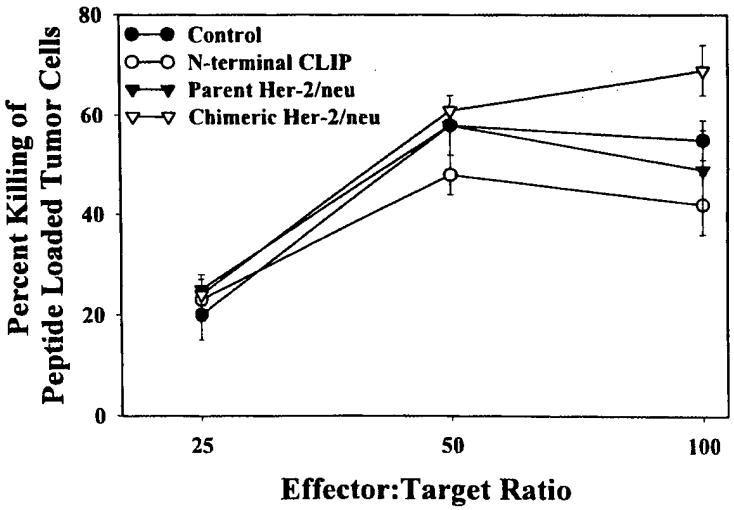

Figure 3 Vaccination with the chimeric peptide elicits an increased frequency of responding lymphocytes

Animals were vaccinated with peptide loaded, irradiated (5000R) tumor cells (parent p1171-1185 and the chimeric construct). Splenic T cells were harvested and limiting dilution cultures established stimulating with antigen presenting cells loaded with either the chimeric construct (A) or the parent Her-2/neu peptide (B). Data represented as a modified limiting dilution graph.

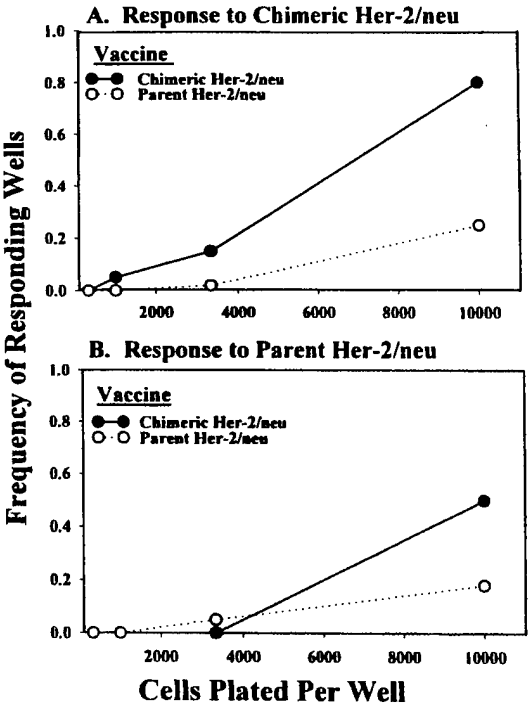

Figure 4 Vaccination with chimeric Her-2/neu peptide loaded tumor cells induced protective antitumor immunity

F344 rats were vaccinated (2X, 14 days apart) with peptide loaded, irradiated (5000R) CRL 1666 tumor cells. Included in the panel of peptides were the parent p1171-1185 peptide, the chimeric construct and the truncated variant of CLIP containing the N-terminal flanking region. Fourteen days following the second vaccination, the animals were challenged with live tumor cells administered intraperitoneally.

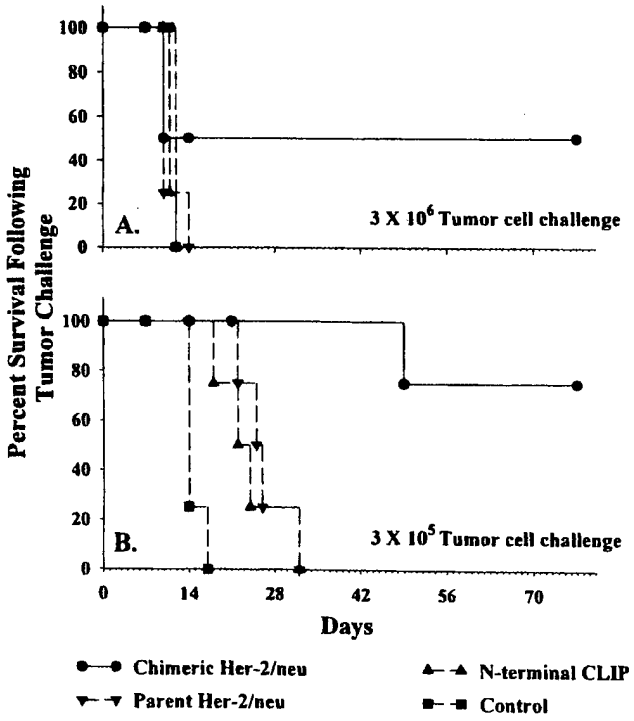

Figure 5A, B Induction of a cytolytic T cell response to a Her-2/neu MHC class I restricted peptide

F344 rats were immunized intradermally (2X, 14 days apart) with dendritic cells (5 x 10^4cells/site, 4 sites) loaded with the MHC class I binding peptide (p554-562) from Her-2/neu. Splenic T lymphocytes were harvested 14 days later and evaluated for their ability to kill peptide loaded PHA blast cells (A) or unmodified CRL 1666 tumor cells (B).

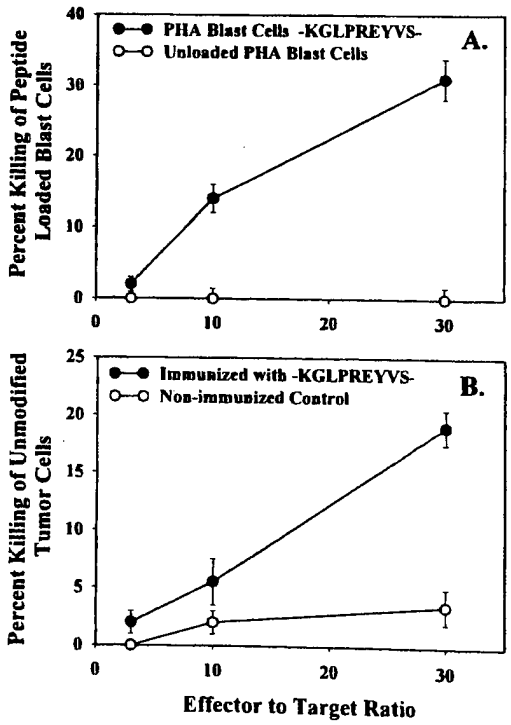

Figure 6 In Vitro targeting of unmodified tumor cells after vaccination with Her-2/neu peptide loaded dendritic cells

F344 rats were vaccinated intradermally twice, 14 days apart with peptide loaded dendritic cells (5×10^4 cells/site, 4 sites). Peptides included the p1171-1185 parent, the chimeric construct, the N-terminal CLIP variant and the MHC class I binding peptide (p544-562) from Her-2/neu. Peptides were loaded singly or in various combinations. Subsequent (14 days) to the last vaccination, splenic T lymphocytes were harvested and evaluated for their ability to kill unmodified CRL 1666 tumor cells.

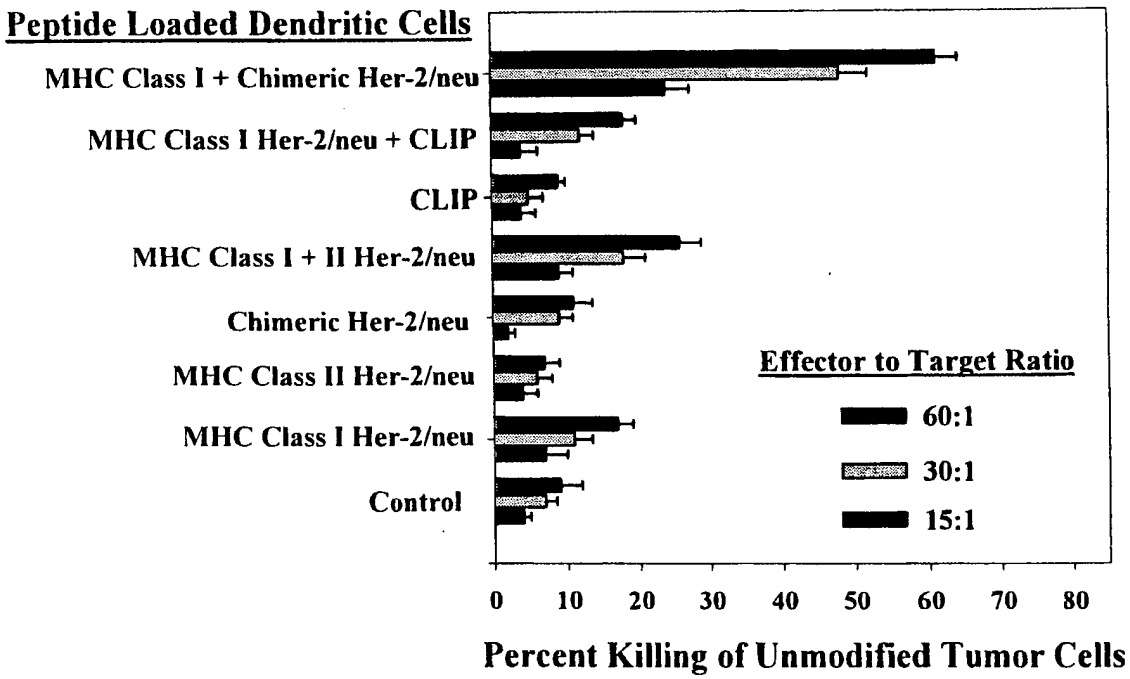

Figure 7 Vaccination with Her-2/neu peptide loaded dendritic cells induces protective antitumor immunity

F344 rats were vaccinated intradermally (2X, 14 days apart) with dendritic cells (5×10^4 cells/site, 4 sites) that were loaded singly or in various combinations with the MHC class I (p554-562) and class II (p1175-1185) Her-2/neu peptides, the chimeric construct or the N-terminal CLIP variant. Fourteen days later, the animals were challenged with live tumor (3×10^5 CRL 1666 cells) administered intraperitoneally.

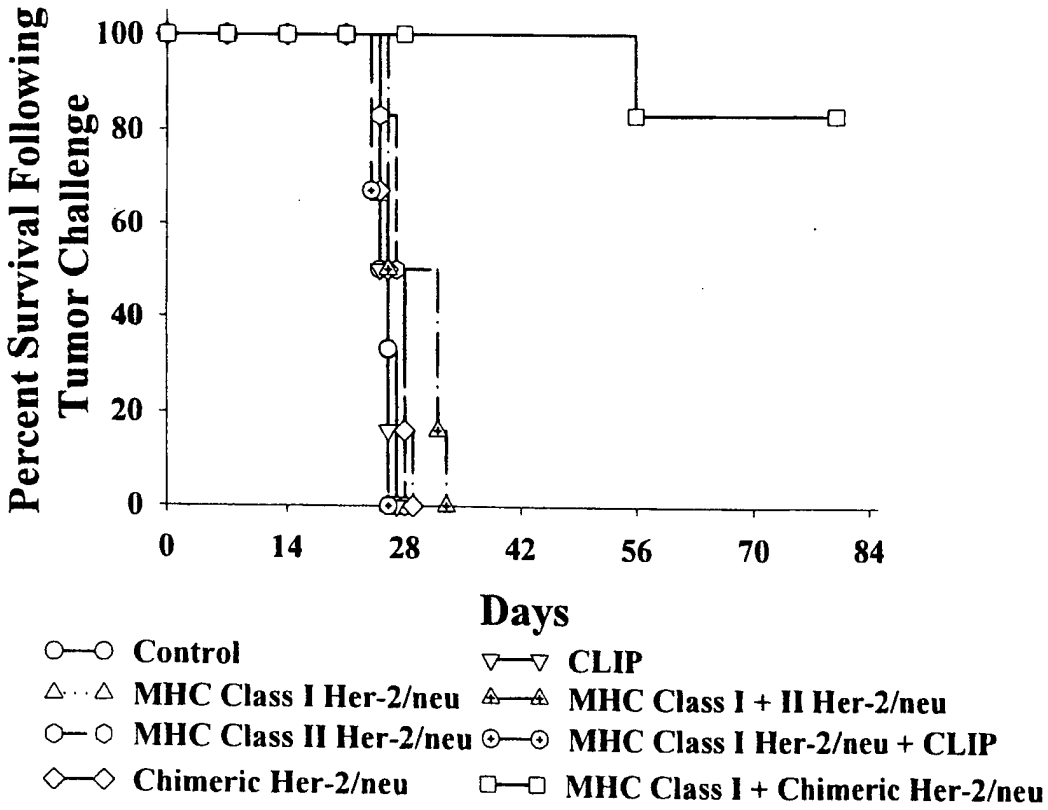

Figure 8 Effective antitumor immunity requires both CD4+ and CD8+ T cells

Animals were vaccinated (2X) intradermally with dendritic cells (5 x 10^4 cells/site, 4 sites) loaded with the MHC class I (p554-562) and the chimeric (p1171-1185) construct. Fourteen days after the last vaccination, the splenic T lymphocytes were harvested and the CD8+ and CD4+ subsets isolated by immunomagnetic bead separation. The cells were adoptively transferred into secondary F344 recipients (unfractionated, isolated CD4+ and CD8+ subsets: 30 x 10^6 cells per recipient: recombined subsets, 15 X 10^6 of each per recipient). Following (1 day) the adoptive transfer, the animals were challenged with 3 X 10^5 viable CRL1666 tumor cells.

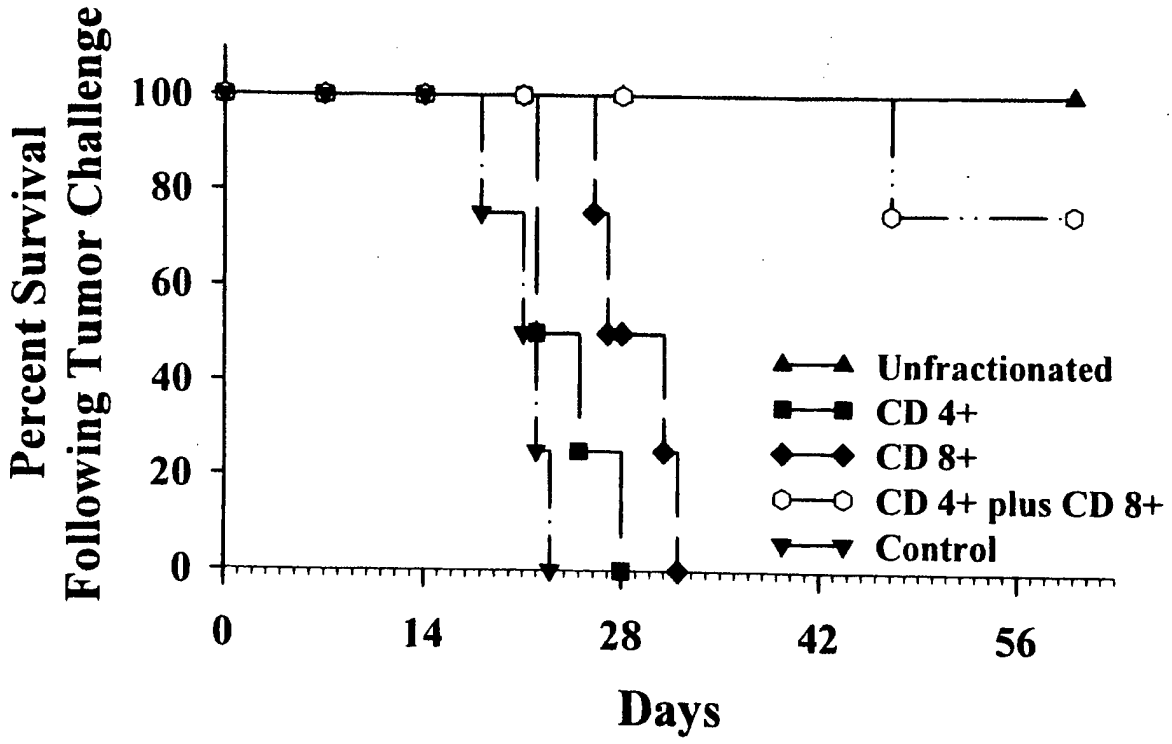

Figure 9 A, B Inhibition of FL-CLIP binding by parent and chimeric Her-2/neu peptides

PHA blast cells were incubated for 2 hours at 4°C with fluoresceinated CLIP (A, 0.3 microMolar: B, 3.0 microMolar) in the presence of graded quantities of the parent p1171-1185 peptide, the chimeric construct or the N-terminal variant of CLIP. The cells were washed prior to flow cytometric analysis on an EPICS IV Coulter flow cytometer evaluating the percent of cells staining with the fluoresceinated CLIP in the presence of the different peptides.

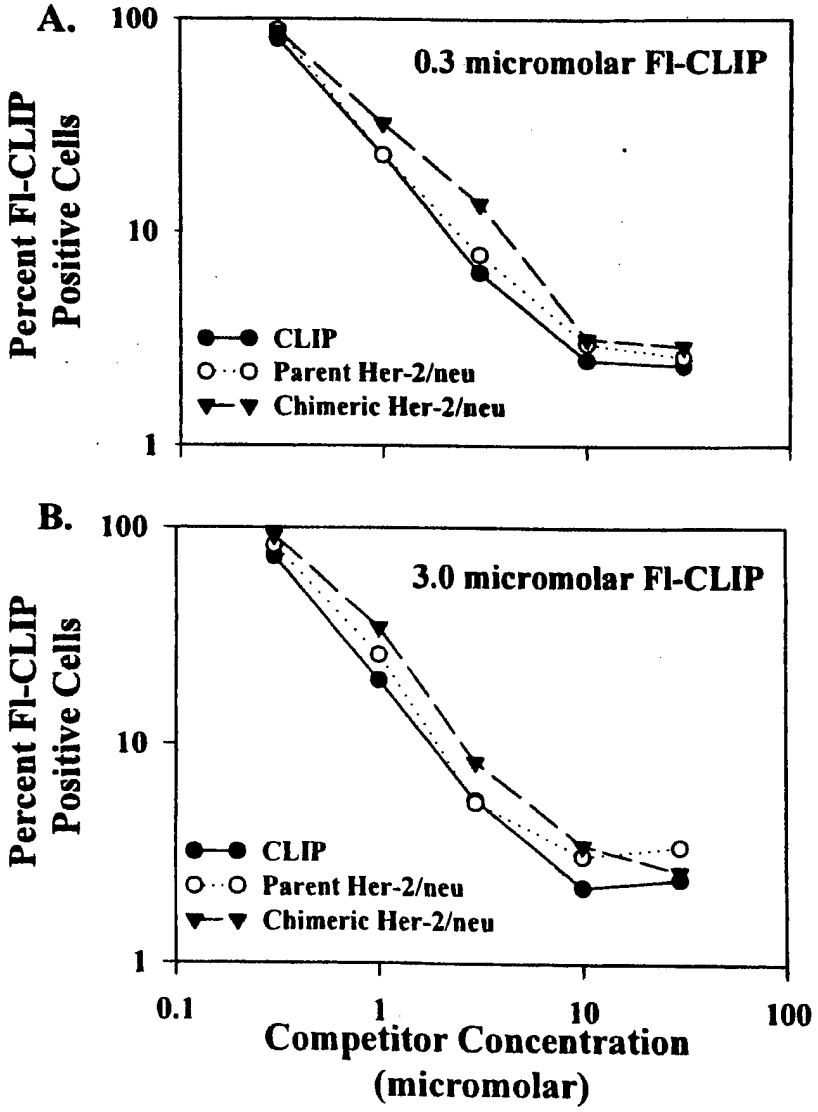

Table 1

Peptide Sequences

Peptide	Sequence
MHC class II - Parent Her-2/neu (p1171-1185)	TLERPKTLSPGKNGV
Chimeric Her-2/neu	KPVSPMTLERPKTLSPGKNGV
N-Terminal CLIP Variant (p86-100)	KPVSPMRMATPLLMRS
MHC class I - Her-2/neu (p554-562)	KGLPREYVS